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(54) Title: HUMAN AKT-3			
(57) Abstract <p>There is disclosed a nucleic acid molecule encoding human Akt-3 protein or a functional equivalent or bioprecursor thereof comprising the amino acid sequence illustrated in Sequence ID No. 3. The human Akt-3 protein itself also forms part of the invention. The nucleic acid molecule and the human Akt-3 protein may themselves be used as medicaments, or in the preparation of medicaments for treating cancer, in their own right or in the form of a pharmaceutically acceptable carrier, diluent or excipient thereof. Further disclosed are methods of identifying agents which influence the activity of a human Akt-3 protein.</p>			
<p>1 GGGAGTCATCATGAGCGATGTTACCATTTGTGAAGAGGTTGGGTCAGAAAGAGGGGAGA 17 61 ATATATAAAAACTGGAGGCCAAGATACCTCTTTTGAAGACAGATGGCTCATTCATAG 27 121 YKKEKPPQDVLKLPYPLNPFSSVA 57 181 KCOLMKTERPKPNNTFIIRCL 77 241 OWTTVIERTFHVDTPPEEREE 97 301 WTEAIOAVADRLOROEEERHM 117 361 NCSPSTSOINDIGEEEMDAST 137 421 THHKRKRKTMNDPFDVLLKLLGKKG 157 481 TFGKVIILVREKASGKYYAMK 177 541 ILLKKEVILIAKDEVAHLLTES 197 601 RVLKNTLRHHPFLTSLKYSFOT 217 661 KDRLLCFVMEYVNGGELFFHL 237 721 AAGACCGTTTGTGTTTGTGATGGAATATGTTAATGGGGCGAGCTGTTTTCATT 257 781 ALDYLHSGKIVVYRDLKLENL 277 841 MLDKDGHIKIDTFGLCKEIGI 297 901 TDAATMKTFCTGTGGCACTCCAGAAATATCTGGCACCAGAGGT 317 961 LEDNDYGRADVWNGLGVMY 337 1021 EMMCGRLPFPYNO DHEKLFEL 357 1081 ILMEDIKFPPTRLSSDAKSL 377 1141 SGLLIKDPNKRLLGGGPD DAK 397 1201 EIMRHHSFSPSGVNWODVYDKK 417 1261 LVPPFPKPOVTSSETDTRYFDE 437 1321 EFTTAQTITITPPEKYDDE DGM 457 1381 AGAATTTACAGCTCAGACTATACAAATACACCACTGAAAATATGATGAGGATGAT 477 1441 DCMRNKZRPHEPDEFSAS 497 1501 TGATCTGGACATCAGCTCTAGCTCTTACACATAGCAGGGGCA</p>			

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C/PRTS

HUMAN AKT-3

The present invention is concerned with cloning and expression of a new human serine/threonine kinase termed "Akt-3" and, in particular, with nucleic acid molecules encoding the Akt-3 protein, the protein itself and compounds which can be used to inhibit cell survival.

A characteristic feature of many cancer cells is their ability to grow independently of adhesion. In contrast, when untransformed endothelial cells are prevented from adhering to the extracellular matrix (ECM), they undergo apoptosis (Frisch & Francis, 1994; Meredith et al., 1993). The process by which normally adherent cells are triggered to undergo apoptosis when they are unable to adhere to ECM has been termed "anoikis" (Frisch & Ruoslahti, 1997) and is an example of the effect on a cell of removal of a survival factor. Changes in signalling by adhesion molecules can lead to resistance to anoikis (Frisch & Ruoslahti, 1997) and this may contribute to the mechanism whereby cancer cells that grow independently of adhesion are able to avoid anoikis.

Akt (also known as protein kinase B (PKB) or "related to A and C protein kinase" (RAC-PK)) is a serine/threonine kinase that has been implicated in regulating cell survival (Khwaja et al., 1997; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Datta et al., 1997; Marte & Downward, 1997). Akt can inhibit apoptosis induced by detachment from ECM (anoikis; Khwaja et al., 1997), as well as by survival factor withdrawal (Kennedy et al., 1997; Ahmed et al., 1997; Dudek et al., 1997; Kauffman-Zeh et al., 1997; Philpott et al., 1997; Crowder & Freeman, 1996; Eves et al., 1998) or irradiation

(Kulik et al., 1997).

Akt comprises an NH₂-terminal pleckstrin homology (PH) domain involved in lipid binding, a kinase domain and a COOH-terminal "tail". Akt is thought to be activated by recruitment to the plasma membrane and subsequent phosphorylation by two upstream kinases, PDK-1 and PDK-2 (reviewed in Coffey et al., 1998; Alessi & Cohen, 1998). The binding of 3-phosphoinositides, generated by phosphatidylinositol 3-kinase (PI 3-kinase), to the PH domain of Akt is believed to promote translocation to the plasma membrane and to facilitate phosphorylation of Akt-1 by PDK-1 at Thr³⁰⁸ (Alessi et al., 1996; Alessi et al., 1997; Stephens et al., 1998) or of Akt-2 at Thr³⁰⁹ (Meier et al., 1997). In addition to phosphorylation of Thr³⁰⁸, full activation requires phosphorylation of the COOH tail at Ser⁴⁷³ in Akt-1 (Alessi et al., 1996) or at Ser⁴⁷⁴ in Akt-2 (Meier et al., 1997). The enzyme responsible for phosphorylation of Ser⁴⁷³/Ser⁴⁷⁴ was originally named PDK-2 but recently the integrin-linked kinase, ILK (Delcommenne et al., 1998) has emerged as a candidate for this function.

Two human isoforms of Akt have been described to date, Akt-1 and Akt-2 (Coffey & Woodgett, 1991; Jones et al., 1991; Cheng et al., 1992). A third isoform, here referred to as Akt-3, has been described in the rat (Konishi et al., 1995). Since this rat Akt-3 possesses an apparently truncated tail and thereby lacks Ser⁴⁷³, its regulation may differ from that of Akt-1 and Akt-2. Both Akt-1 and Akt-2 are expressed widely, although the expression of Akt-2 is most prominent in insulin-responsive tissues, such as liver and skeletal muscle (Konishi et al., 1994; Altomare et al., 1995). Akt-1 and Akt-2 are activated by insulin in rat adipocytes, hepatocytes and skeletal muscle. In contrast, Akt-3 does not appear to be strongly activated by insulin in

these tissues (Walker et al., 1998). The role of the various Akt isoforms in insulin signalling may limit the utility of compounds that inhibit Akt-1 or Akt-2 activity as such agents may induce symptoms observed in patients with diabetes. We hypothesized that this problem may be avoided by using selective inhibitors of Akt-3 and this prompted us to identify the human analogue of rat Akt-3.

The present inventors have now identified and characterised a nucleic acid molecule that encodes the human isoform of Akt-3. Significantly, human Akt-3 possesses a COOH-terminal tail that contains an amino acid residue analogous to Ser⁴⁷³/Ser⁴⁷⁴ previously implicated in the activation of Akt-1/Akt-2, but absent in the rat Akt-3 protein.

Therefore, there is provided by a first aspect of the present invention a nucleic acid molecule encoding human Akt-3 or a functional equivalent, derivative or bioprecursor thereof, comprising the amino acid sequence illustrated in Figure 2. Preferably, the molecule is a DNA molecule and even more preferably a cDNA molecule, and even more preferably comprises the sequence of nucleotides illustrated in Figure 1. Also provided by this aspect of the invention is a nucleic acid molecule capable of hybridising to the molecule according to the invention under high stringency conditions.

Stringency of hybridisation as used herein refers to conditions under which polynucleic acids are stable. The stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. T_m can be approximated by the formula:

$$81.5^{\circ}\text{C} + 16.6(\log_{10}[\text{Na}^+]) + 0.41 (\% \text{G\&C}) - 600/1$$

wherein l is the length of the hybrids in nucleotides. T_m decreases approximately by 1-1.5°C with every 1% decrease in sequence homology.

5 The term "stringency" refers to the hybridisation conditions wherein a single-stranded nucleic acid joins with a complementary strand when the purine or pyrimidine bases therein pair with their corresponding base by hydrogen bonding. High stringency conditions
10 favour homologous base pairing whereas low stringency conditions favour non-homologous base pairing.

"Low stringency" conditions comprise, for example, a temperature of about 37°C or less, a formamide
15 concentration of less than about 50%, and a moderate to low salt (SSC) concentration; or, alternatively, a temperature of about 50°C or less, and a moderate to high salt (SSPE) concentration, for example 1M NaCl.

20 "High stringency" conditions comprise, for example, a temperature of about 42°C or less, a formamide concentration of less than about 20%, and a low salt (SSC) concentration; or, alternatively, a temperature of about 65°C, or less, and a low salt (SSPE)
25 concentration. For example, high stringency conditions comprise hybridization in 0.5 M NaHPO_4 , 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C (Ausubel, F.M. et al. Current Protocols in Molecular Biology, Vol. I, 1989; Green Inc. New York, at
30 2.10.3).

"SSC" comprises a hybridization and wash solution. A stock 20X SSC solution contains 3M sodium chloride, 0.3M sodium citrate, pH 7.0.
35

"SSPE" comprises a hybridization and wash solution. A 1X SSPE solution contains 180 mM NaCl, 10mM NaH_2PO_4

and 1 mM EDTA, pH 7.4.

5 The nucleic acid capable of hybridising to nucleic acid molecules according to the invention will generally be at least 85%, preferably at least 90% and even more preferably at least 95% homologous to the nucleotide sequences according to the invention.

10 The DNA molecules according to the invention may, advantageously, be included in a suitable expression vector to express polypeptides encoded therefrom in a suitable host.

15 The present invention also comprises within its scope proteins or polypeptides encoded by the nucleic acid molecules according to the invention or a functional equivalent, derivative or bioprecursor thereof.

20 An expression vector according to the invention includes a vector having a nucleic acid according to the invention operably linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of said DNA fragments. The term "operably linked" refers to a juxta position wherein
25 the components described are in a relationship permitting them to function in their intended manner. Such vectors may be transformed into a suitable host cell to provide for expression of a polypeptide according to the invention. Thus, in a further
30 aspect, the invention provides a process for preparing polypeptides according to the invention which comprises cultivating a host cell, transformed or transfected with an expression vector as described above under conditions to provide for expression by
35 the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

The vectors may be, for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of said nucleotide and optionally a regulator of the promoter.

5 The vectors may contain one or more selectable markers, such as, for example, ampicillin resistance.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and

10 transcription initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a

15 eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained

20 commercially or assembled from the sequences described by methods well known in the art.

A nucleic acid molecule according to the invention may be inserted into the vectors described in an antisense

25 orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense nucleic acids may be produced by synthetic means.

In accordance with the present invention, a defined

30 nucleic acid includes not only the identical nucleic acid but also any minor base variations including in particular, substitutions in bases which result in a synonymous codon (a different codon specifying the same amino acid residue) due to the degenerate code in

35 conservative amino acid substitutions. The term "nucleic acid sequence" also includes the complementary sequence to any single stranded sequence

given regarding base variations.

The present invention also advantageously provides nucleic acid sequences of at least approximately 10
5 contiguous nucleotides of a nucleic acid according to the invention and preferably from 10 to 120, and even more preferably from 10 to approximately 50 nucleotides. These sequences may, advantageously be used as probes or primers to initiate replication, or
10 the like. Such nucleic acid sequences may be produced according to techniques well known in the art, such as by recombinant or synthetic means. They may also be used in diagnostic kits or the like for detecting the presence of a nucleic acid according to the invention.
15 These tests generally comprise contacting the probe with the sample under hybridising conditions and detecting for the presence of any duplex or triplex formation between the probe and any nucleic acid in the sample.

20 According to the present invention these probes may be anchored to a solid support. Preferably, they are present on an array so that multiple probes can simultaneously hybridize to a single biological
25 sample. The probes can be spotted onto the array or synthesised *in situ* on the array. (See Lockhart et al., Nature Biotechnology, vol. 14, December 1996 "Expression monitoring by hybridisation to high density oligonucleotide arrays". A single array can
30 contain more than 100, 500 or even 1,000 different probes in discrete locations.

Advantageously, the nucleic acid sequences, according to the invention may be produced using such
35 recombinant or synthetic means, such as for example using PCR cloning mechanisms which generally involve making a pair of primers, which may be from

approximately 10 to 50 nucleotides to a region of the gene which is desired to be cloned, bringing the primers into contact with mRNA, cDNA, or genomic DNA from a human cell, performing a polymerase chain
5 reaction under conditions which bring about amplification of the desired region, isolating the amplified region or fragment and recovering the amplified DNA. Generally, such techniques as defined herein are well known in the art, such as described in
10 Sambrook et al (Molecular Cloning: a Laboratory Manual, 1989).

The nucleic acids or oligonucleotides according to the invention may carry a revealing label. Suitable
15 labels include radioisotopes such as ^{32}P or ^{35}S , enzyme labels or other protein labels such as biotin or fluorescent markers. Such labels may be added to the nucleic acids or oligonucleotides of the invention and may be detected using known techniques per se.

20 A further aspect of the invention comprises human Akt-3 or a functional equivalent, derivative or bioprecursor thereof, comprising an amino acid sequence as illustrated in Figure 2.

25 The polypeptide designated human Akt-3 according to the invention includes all possible amino acid variants encoded by the nucleic acid molecule according to the invention including a polypeptide
30 encoded by said molecule and having conservative amino acid changes. Polypeptides according to the invention further include variants of such sequences, including naturally occurring allelic variants which are substantially homologous to said polypeptides. In
35 this context, substantial homology is regarded as a sequence which has at least 90% amino acid homology with the polypeptides encoded by the nucleic acid

molecules according to the invention and even more preferably at least 95% amino acid homology.

5 The nucleic acid molecule or the human Akt-3 according to the invention may, advantageously, be used as a medicament or in the preparation of a medicament, for treating disease associated with Akt-3 activity such as, cancer or the like.

10 Advantageously, the nucleic acid molecule or the polypeptide according to the invention may be provided in a pharmaceutical composition together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

15 The present invention is further directed to inhibiting Akt-3 *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation
20 or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the mature protein sequence, which encodes for the protein of the present invention, is used to design an antisense RNA
25 oligonucleotide of from 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix - see Lee et al. Nucl. Acids Res., 6:3073 (1979); Cooney et al., Science, 241:456 (1988);
30 and Dervan et al., Science, 251: 1360 (1991), thereby preventing transcription and the production of Akt-3. The antisense RNA oligonucleotide hybridises to the mRNA *in vivo* and blocks translation of an mRNA molecule into the Akt-3 (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as
35 Antisense Inhibitors of Gene Expression, CRC Press,

Boca Raton, FL (1998)).

Alternatively, the oligonucleotide described above can be delivered to cells by procedures in the art such that the anti-sense RNA or DNA may be expressed *in vivo* to inhibit production of Akt-3 in the manner described above.

Antisense constructs to Akt-3, therefore, may inhibit the survival of the cell and prevent further cancer or tumour growth.

According to a further aspect of the invention there is also provided a transgenic cell, tissue or organism comprising a transgene capable of expressing human Akt-3 protein according to the invention. The term "transgene capable of expression" as used herein means a suitable nucleic acid sequence which leads to expression of human Akt-3 or human proteins having the same function and/or activity. The transgene, may include, for example, genomic nucleic acid isolated from human cells or synthetic nucleic acid, including DNA integrated into the genome or in an extrachromosomal state. Preferably, the transgene comprises the nucleic acid sequence encoding the proteins according to the invention as described herein, or a functional fragment of said nucleic acid. A functional fragment of said nucleic acid should be taken to mean a fragment of the gene comprising said nucleic acid coding for the proteins according to the invention or a functional equivalent, derivative or a non-functional derivative such as a dominant negative mutant, or bioprecursor of said proteins. For example, it would be readily apparent to persons skilled in the art that nucleotide substitutions or deletions may be used using routine techniques, which do not affect the protein sequence encoded by said

nucleic acid, or which encode a functional protein according to the invention.

5 Human Akt-3 protein expressed by said transgenic cell, tissue or organism or a functional equivalent or bioprecursor of said protein also form part of the present invention.

10 Antibodies to human Akt-3 may, advantageously, be prepared by techniques which are known in the art. For example, polyclonal antibodies may be prepared by inoculating a host animal, such as a mouse, with human Akt-3 according to the invention or an epitope thereof and recovering immune serum. Monoclonal antibodies may
15 be prepared according to known techniques such as described by Kohler R. and Milstein C., Nature (1975) 256, 495-497.

20 Antibodies according to the invention may also be used in a method of detecting for the presence of human Akt-3 according to the invention, which method comprises reacting the antibody with a sample and identifying any protein bound to said antibody. A kit may also be provided for performing said method which
25 comprises an antibody according to the invention and means for reacting the antibody with said sample.

30 Proteins which interact with the polypeptide of the invention may be identified by, for example, investigating protein-protein interactions using the two-hybrid vector system first proposed by Chien et al (1991). Proc. Natl. Acad. Sci. USA 88: 9578-9582.

35 This technique is based on functional reconstitution in vivo of a transcription factor which activates a reporter gene. More particularly the technique comprises providing an appropriate host cell with a

DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA binding domain and an activating domain, expressing in the host cell a first hybrid DNA sequence encoding a first fusion of a fragment or all of a nucleic acid sequence according to the invention and either said DNA binding domain or said activating domain of the transcription factor, expressing in the host at least one second hybrid DNA sequence, such as a library or the like, encoding putative binding proteins to be investigated together with the DNA binding or activating domain of the transcription factor which is not incorporated in the first fusion; detecting any binding of the proteins to be investigated with a protein according to the invention by detecting for the presence of any reporter gene product in the host cell; optionally isolating second hybrid DNA sequences encoding the binding protein.

An example of such a technique utilises the GAL4 protein in yeast. GAL4 is a transcriptional activator of galactose metabolism in yeast and has a separate domain for binding to activators upstream of the galactose metabolising genes as well as a protein binding domain. Nucleotide vectors may be constructed, one of which comprises the nucleotide residues encoding the DNA binding domain of GAL4. These binding domain residues may be fused to a known protein encoding sequence, such as for example the nucleic acids according to the invention. The other vector comprises the residues encoding the protein binding domain of GAL4. These residues are fused to residues encoding a test protein. Any interaction between polypeptides encoded by the nucleic acid according to the invention and the protein to be tested leads to transcriptional activation of a reporter molecule in a GAL-4 transcription deficient

yeast cell into which the vectors have been transformed. Preferably, a reporter molecule such as β -galactosidase is activated upon restoration of transcription of the yeast galactose metabolism genes.

5

A further aspect of the invention provides a method of identifying compounds which selectively inhibit human Akt-3 mediated promotion of cell survival said method comprising i) providing a cell transformed with an expression vector activating the Akt-3 pathway which cell survives in the presence or absence of a survival factor compared to a control cell which has not been transformed with said vector and will die in the absence of said survival factor ii) contacting said cells with a test compound following removal of said cells from said survival factors, wherein death of said transformed cell is indicative of selective inhibition of said compound on the survival promoting human Akt-3 pathway.

20

Alternatively, the survival promoting activity of Akt-3 could be assessed by i) providing a cell transformed with an expression vector activating the Akt-3 pathway in addition to a control cell which has not been transformed with said vector, ii) contacting each of said cells with a death inducing agent, whereby death of said control cell and survival of said transformed cell is indicative of the survival promoting activity of the activated Akt-3 pathway, iii) subsequently contacting said transformed cell without removal of said death inducing agent, with a test compound, wherein death of said cell is indicative of selective inhibition of said compound on the survival promoting human Akt-3 pathway.

35

In a further aspect the present invention provides methods to identify agents that affect the activity of

the human Akt-3 protein, comprising contacting said protein with a substrate, regulatory molecule or surrogate thereof and monitoring the interaction with the test substance using standard phosphorylation or binding assays well known in the art.

Compounds which are identified according to this aspect of the invention in addition to antibodies to the human Akt-3 may, advantageously, be utilised as a medicament or alternatively in the preparation of a medicament for treating diseases associated with expression of human Akt-3 protein according to the invention.

A further aspect of the invention provides a pharmaceutical composition comprising any of a compound, an antisense molecule or an antibody according to the invention together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

The antisense molecules or indeed the compounds identified as agonists or antagonists of the nucleic acids or polypeptides according to the invention may be used in the form of a pharmaceutical composition, which may be prepared according to procedures well known in the art. Preferred compositions include a pharmaceutically acceptable vehicle or diluent or excipient, such as for example, a physiological saline solution. Other pharmaceutically acceptable carriers including other non-toxic salts, sterile water or the like may also be used. A suitable buffer may also be present allowing the compositions to be lyophilized and stored in sterile conditions prior to reconstitution by the addition of sterile water for subsequent administration. Incorporation of the polypeptides of the invention into a solid or semi-

solid biologically compatible matrix may be carried out which can be implanted into tissues requiring treatment.

- 5 The carrier can also contain other pharmaceutically acceptable excipients for modifying other conditions such as pH, osmolarity, viscosity, sterility, lipophilicity, solubility or the like.
- 10 Pharmaceutically acceptable excipients which permit sustained or delayed release following administration may also be included.

15 The polypeptides, the nucleic acid molecules or compounds according to the invention may be administered orally. In this embodiment they may be encapsulated and combined with suitable carriers in solid dosage forms which would be well known to those skilled in the art.

20 As would be well known to those of skill in the art, the specific dosage regime may be calculated according to the body surface area of the patient or the volume of body space to be occupied, dependent upon the

25 particular route of administration to be used. The amount of the composition actually administered will, however, be determined by a medical practitioner, based on the circumstances pertaining to the disorder to be treated, such as the severity of the symptoms,

30 the composition to be administered, the age, weight, and response of the individual patient and the chosen route of administration.

The present invention may be more clearly understood

35 with reference to the following example which is purely exemplary and the accompanying drawings wherein:

Figure 1 is an illustration of the cDNA sequence and deduced amino acid sequence of human Akt-3. The Akt-3 coding sequence and parts of the 5' and 3' untranslated regions are shown and numbered in the left hand column. The deduced amino acid sequence of the Akt-3 protein is shown above the corresponding DNA sequence and is numbered in the right hand column. The two amino acid residues that are presumed to be phosphorylated upon activation of Akt-3 (Thr³⁰⁵ and Ser⁴⁷²) are in bold and marked with an asterisk. The COOH-terminal part of the human Akt-3 protein that differs with the rat homologue is underlined.

Figure 2 is an alignment of the deduced amino acid sequences for human Akt-1, Akt-2 and Akt-3. The sequences were aligned using the ClustalW alignment program (EMBL, Heidelberg, Germany). Amino acid residues conserved between all three proteins are included in the black areas. Residues conserved between only two of the sequences are shaded in grey. Amino acid residues are numbered in the right hand column. The conserved Thr and Ser residues that are presumed to be phosphorylated upon activation are marked with an asterisk above the sequence.

Figure 3 is an illustration of phosphorylation of histone H2B by Akt-3 variants. (A) Akt-3 was expressed as a GST fusion protein in *E. Coli*. To assess hAkt-3 activity, Histone H2B was incubated with GST-Akt-3 and GST-Akt-3 variants for the indicated time and the extent of phosphorylation assessed after SDS-PAGE. The variants of Akt-3 are designated: W.T., wild type; T305D, Thr³⁰⁵ mutated to Asp; S472D, Ser⁴⁷² mutated to Asp; T305D, S472D, both Thr³⁰⁵ and Ser⁴⁷² mutated to Asp. No significant phosphorylation was observed when GST was used in place of GST-Akt. The results are the mean (\pm s.e.m.; n = 3 to 6) and are expressed relative to

the extent of phosphorylation of H2B catalysed by T305D, S472D hAkt-3 after 45 minutes. Insert, The purity of the purified GST (lane 1), wild-type Akt-3 (lane 2), T305D Akt-3 (lane 3), S472D Akt-3 (lane 4) or T305D/S472D Akt-3 (lane 5) was assessed by SDS-PAGE and by Coomassie blue staining. (B) HEK-293 cells were transfected with either vector (lanes 1 & 2) or Akt-3 (lanes 3 and 4) AKT-3T305A (lanes 5 & 6) or Akt-3 S472A (lanes 7 and 8) and either treated with buffer (lanes 1, 3 5 and 7) or IGF-1 (50 ng/ml; lanes 2, 4, 6 and 8). Akt-3 was immunoprecipitated with antibody 3F10 (anti-HA tag). Samples were analysed by blotting for the HA-tag (upper panel) or with a phosphospecific antibody which recognises phosphorylated ser⁴⁷² (lower panel). (C) Akt activity in HA-immuno-precipitates from samples prepared as described above was assessed by measuring phosphorylation of a peptide substrate (Crosstide). The results are expressed as the increase in activity compared to unstimulated cells transfected with empty vector (mean \pm s.e.m., n=7).

Figure 4 is an illustration of inhibition of Akt-3 by staurosporine and R0 31-8220. Histone H2B was treated with Akt-3 (T305D,S472D variant) in the presence of the indicated concentrations of either staurosporine or R0 31-8220. After 30 minutes, the reaction was terminated and the extent of H2B phosphorylation quantified on a phosphorimager following SDS-PAGE. The results (mean \pm s.e.m., n=3) are expressed as relative to (%) the phosphorylation observed in the presence of solvent (control, "C").

Figure 5 is an illustration of chromosomal localisation of human Akt-3. (A) Diagram of FISH mapping results of Akt-3. Each dot represents the double FISH signals detected on human chromosome 1, region q43- q44. (B) Example of FISH mapping of Akt-3.

The left panel shows the FISH signals on chromosome 1. The right panel shows the same mitotic figure stained with 4',6- diamidino-2-phenylindole to identify chromosome 1.

5

Figure 6 is an illustration of expression of Akt-3 in different human tissues. (A) Northern blot analysis of tissue expression of Akt-3. The expression of hAkt-3 mRNA in different human tissues was assessed using a probe corresponding to the 3' untranslated region of hAkt-3 to analyse a blot of human polyA⁺ RNA ("Multiple Tissue Northern"). Human β -actin was used as a control to confirm equal loading of the lanes (data not shown). (B) and (C) RT-PCR analysis of tissue expression of Akt-3. RT-PCR analyses were performed on cDNA from different human tissues (B) and from different tumor cell lines (C) using primers specific for human Akt-3 or G3PDH (control) for the indicated number of PCR cycles. Bands of the expected size (425 bp for Akt-3 and 1 kb for G3PDH) are visible on the gels. The images from the ethidium bromide stained 1.2% agarose gels were inverted for clarity using the EagleSight software (Stratagene). The results from similar PCR reactions performed for 25, 30 or 35 cycles are not shown but indicated that the results from this figure are in the linear range of amplification. Caco-2 = colorectal adenocarcinoma; T-84 = colorectal carcinoma; MCF-7 = breast adenocarcinoma; T-47D = breast ductal gland carcinoma; HT1080 = bone fibrosarcoma; SaOS-2 = osteosarcoma; SK-N-MC = neuroblastoma; HepG2 = hepatoblastoma; JURKAT = T-cell leukemia.

Figure 7 is an illustration of the results obtained by scintillation counting in a scintillation proximity assay to identify agents that modulate the activity of Akt-3 activity.

Figure 8 is an illustration of the results obtained from an Akt-3 filter assay to identify agents that modulate activity of Akt-3.

5 MATERIALS AND METHODS

Oligonucleotide synthesis and DNA sequence determination

10 All primers were obtained from Eurogentec, Seraing, Belgium. Insert-specific sequencing primers (15- and 16-mers) were designed by visual inspection of the DNA sequences. DNA was prepared on Qiagen-tip-20 columns or on Qiaquick spin columns (Qiagen GmbH, Düsseldorf,
15 Germany) and recovered from the spin columns in 30 μ l Tris/EDTA-buffer (10mM TrisHCl pH 7.5, 1 mM EDTA (sodium salt)). Sequencing reactions were performed using BigDye™ Terminator Cycle Sequencing Ready Reaction kits (Perkin Elmer, ABI Division, Foster
20 City, CA, USA) and were run on an Applied Biosystems 377 DNA sequencer (Perkin Elmer, ABI Division, Foster City, CA, USA).

Molecular cloning of human Akt-3.

25 Using the rat RAC-PKy sequence (Konishi et al, 1995; GenBank acc. No. D49836) as a query sequence, a BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990) search was carried out in the WashU Merck
30 expressed sequence tag (EST) database (Lennon et al., 1996) and in the proprietary LifeSeq™ human EST database (Incyte Pharmaceuticals Inc, Palo Alto, CA, USA). Several human EST clones with high similarity to the rat RAC-PKy were identified. One EST sequence
35 (Incyte accession number 2573448) derived from a hippocampal cDNA library, contained part of the coding

sequence including the putative methionine start codon (ATG) and part of the 5' untranslated region. The start codon was surrounded by a Kozak consensus sequence for translation start and an in-frame stop codon was present at positions -6 to -3. Based on this 239 bp sequence, oligonucleotide sense primers were synthesised for 3' rapid amplification of cDNA ends (3' RACE) experiments: Akt-3sp1 = 5'-ACC ATT TCT CCA AGT TGG GGG CTC AG-3' and Akt-3sp2 = 5'GGG AGT CAT CAT GAG CGA TGT TAC C-3'. 3'RACE experiments were performed on human fetal brain or human cerebellum Marathon-Ready™ cDNA (Clontech Laboratories, Palo Alto, CA, USA) according to manufacturer's instructions using Akt-3sp1/race-ap1 as primers in the primary PCR and Akt-3sp2/race-ap2 in the nested PCR. Resulting PCR fragments were cloned and sequenced. This extended the Akt-3 coding sequence by 916 bp, but the novel sequence did not include an in-frame stop codon. A second round of 3' RACE amplification was performed on human brain Marathon Ready™ cDNA using sense primers based on the sequence obtained in the first round (Akt-3sp3 = 5'CAC TCC AGA ATA TCT GGC ACC AGA GG-3' and Akt-3sp4 = 5'CTA TGG CCG AGC AGT AGA CTG GTG G-3') in combination with race-ap1 and race-ap2, respectively. The sequence obtained included an in-frame stop codon and the 3' untranslated sequence up to the poly(A) tail. Antisense primers were designed based on the 3' untranslated region (Akt-3ap4 = 5'-TGC CCC TGC TAT GTG TAA GAG CTA GG-3' and Akt-3ap5 = 5' AAG AGC TAG GAC TGG TGA TGT CCA GG-3') and the complete Akt-3 coding sequence was amplified from human hippocampal cDNA using Akt-3sp1/Akt-3ap4 (primary PCR) and Akt-3sp2/Akt-3ap5 (nested PCR) as primers. The resulting 1200 bp PCR fragment was then cloned in the TA-cloning vector pCR2.1 (original TA cloning kit, Invitrogen BV, Leek, The Netherlands) and the inserts of several clones were completely sequenced. One

clone containing an insert with the confirmed sequence (hAkt-3/pCR2.1) was used for subsequent subcloning to the mammalian expression vector pcDNA-3 (Invitrogen), yielding construct hAkt-3/pcDNA-3. In order to make a
5 construct coding for a COOH-terminal tagged Akt-3 protein, a fragment of 553 bp was amplified from plasmid Akt-3/pcDNA-3 using an antisense primer incorporating a XhoI restriction site and the sequence coding for a hemagglutinin (HA) tag (YPYDVPDYA) after
10 amino acid 479 of the Akt-3 sequence. This fragment was recloned into plasmid hAkt-3/pcDNA-3 using BstEII and XhoI restriction sites yielding construct HA-hAkt-3/pcDNA-3.

15 **Constructs and mutants for *E. coli* expression of Akt-3.**

In order to express the human Akt-3 protein in *E. coli*, the complete Akt-3 coding sequence was amplified
20 from plasmid hAkt-3/pCR2.1 using primers introducing a EcoRI restriction site and a XhoI restriction site at the 5' and 3' ends, respectively. This PCR fragment was cloned as a EcoRI/XhoI fragment in vector pGEX-4T-3 (Amersham Pharmacia Biotech, Uppsala, Sweden) yielding
25 construct hAKT-3(WT)/pGEX-4T-3, and the sequence of the insert was confirmed by sequence analysis.

Mutants of this construct were made using the Quickchange site-directed mutagenesis kit (Stratagene,
30 La Jolla, CA, USA) according to the manufacturer's instructions. The T305D mutant (construct hAKT-3(T305D)/pGEX-4T-3) was created by mutating ACA at position 923-925 to GAC, resulting in a Thr³⁰⁵ to Asp mutation in the resulting protein. The S472D mutant
35 (construct hAKT-3(S472D)/pGEX-4T-3) was created by changing TC at position 1404-1405 to GA using PCR with

a long antisense primer incorporating the change, resulting in a Ser⁴⁷² to Asp mutation in the resulting protein. A double mutant was also constructed by site-directed mutagenesis on hAKT-3(S472D)/pGEX-4T-3 and
5 contained both these mutations (construct hAKT-3(T305D/S472D)/pGEX-4T-3). The inserts of all resulting constructs were confirmed by complete sequence analysis. The fusion proteins resulting from expression of these constructs in *E. coli* contain a
10 GST moiety coupled to the NH₂-terminus of the human Akt-3 sequence.

Expression in Cos-7 cells and HEK-293 cells

15 Akt-3 was transiently expressed in Cos-7 by calcium phosphate transfection of the cells with the construct HA-hAkt-3/pcDNA-3. The cells were stimulated with 10 ng/ml IGF-1 for 30 minutes, lysed and Akt-3 immunoprecipitated with mAb 12CA5. Akt-3 activity was
20 assessed as described below.

For expression in HEK-293 cells, cells were transfected with pCDNA-3 Akt-3 constructs as described previously (Alessi et al 1996). After stimulation
25 with IGF, the cells were lysed (Alessi et al 1996) and HA-Akt immunoprecipitated with antibody 3F10 (Roche Molecular Biochemicals). Akt activity was assessed in immune complexes by measuring phosphorylation of a peptide substrate (Crosstide) in the presence of 1 μ M
30 PKI (PKA inhibitor) and 1 μ M GF 109302X (PKC inhibitor) as described.

Expression and assay of wild-type and mutant Akt-3 in *E. coli*.

35

The pGEX expression constructs were transformed into

E. coli strain BL21 DE3 and GST-fusion proteins of wild-type and mutated Akt-3 were purified on glutathione sepharose according to the manufacturer's instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). The protein eluted from the beads was stored in 50% glycerol at -20°C. Akt activity was assessed by incubating 0.8 µg of the purified enzyme for 30 minutes at room temperature (unless otherwise indicated) in a buffer containing 10 mM HEPES, 10 mM MgCl₂, 1 mM DTT, 0.1 mg/ml histone H2B at pH 7.0, in a total volume of 25 µl and containing 10 µCi [γ-³²P]-ATP (6000 Ci/mmol). Initial experiments indicated that the reaction was linear with time for at least 45 minutes. The reaction was stopped by the addition of 25 µl sample buffer for SDS-PAGE. The results were quantified on a phosphorimager following SDS-PAGE on a 15% (w/v) acrylamide gel.

Chromosomal mapping studies

Chromosomal mapping studies were carried out by SeedNA Biotech Inc, Toronto, Canada using fluorescent *in situ* hybridisation (FISH) analysis essentially as described (Heng et al., 1992; Heng & Tsui, 1993). Briefly, human lymphocytes were cultured at 37°C for 68-72 h before treatment with 0.18 mg/ml 5-bromo-2'-deoxyuridine (BrdU) to synchronize the cell cycle in the cell population. The synchronized cells were washed and recultured at 37°C for 6 h. Cells were harvested and slides were prepared using standard procedures including hypotonic treatment, fixation and air-drying. A cDNA probe for Akt-3 (1.44 kb EcoRI fragment of clone hAkt-3/pcDNA-3) was biotinylated and used for FISH detection. Slides were baked at 55°C for 1 h, treated with RNase and denatured in 70% (v/v) formamide in 2x NaCl/Cit (0.3 M NaCl, 0.03 M disodium

citrate, pH 7.0) for 2 min at 70°C followed by dehydration in ethanol. Probes were denatured prior to loading on the denatured chromosomal slides. After overnight hybridisation, slides were washed and FISH signals and the 4',6-diamidino-2-phenylindole banding pattern were recorded separately on photographic film, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposition of FISH signals with 4,6-diamidino-2-phenylindole banded chromosomes (Heng & Tsui, 1993).

Northern blot analysis.

Northern blots containing 2 µg of poly(A)-rich RNA derived from different human tissues (Clontech Laboratories, Palo Alto, CA, USA) were hybridised according to the manufacturer's instructions with a α-³²P-dCTP random-priming labelled (HighPrime kit, Boehringer Mannheim) 454 bp NotI-XbaI Akt-3 fragment (nucleotides 1404 to 1857) corresponding to part of the 3' untranslated sequence.

Reverse transcription (RT)-PCR analysis

Oligonucleotide primers were designed for the specific PCR amplification of a fragment from Akt-3. These primers were Akt-3sp2 = 5'-GGG AGT CAT CAT GAG CGA TGT TAC C-3' (sense primer) and Akt-3ap1 = 5'- GGG TTG TAG AGG CAT CCA TCT CTT CC -3' (antisense primer), yielding a 425 bp product. PCR amplifications for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were performed on the same cDNA samples as positive controls using G3PDH primers 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3' (sense primer) and 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3' (antisense primer), yielding a 1000

- 25 -

bp fragment. These primers were used for PCR amplifications on Multiple Tissue cDNA panels (Clontech Laboratories) and on cDNA prepared from tumor cell lines. For the preparation of tumor cell cDNA, cells were homogenised and total RNA prepared using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. 1 μ g of total RNA was reverse transcribed using oligo(dT)₁₅ as a primer and 50 U of Expand™ Reverse Transcriptase (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. PCR reactions with Akt-3-specific or G3PDH-specific primers were then performed on 1 μ l of cDNA. Images of the ethidium bromide stained gels were obtained using the Eagle Eye II Video system (Stratagene, La Jolla, CA, USA) and PCR bands analysed using the EagleSight software.

Assays to identify agents that modulate the activity of Akt-3

To identify agents that modulate the activity of Akt-3, SPA (scintillation proximity assay) and filter assays for Akt-3 activity were developed.

SPA assays were performed at 25°C for 3 hrs in the presence of 25mM Hepes, pH 7.0, containing 15 mM MgCl₂, 1 mM DTT. Each assay was performed in a 100 μ l reaction volume containing 111nM GST-AKT-3 (diluted in 25 mM Hepes, pH 7.0, containing 15 mM MgCl₂, 1 mM DTT), 0.75 μ M Biotinylated Histone H2B, 2nM [γ -³²P]-ATP and any agents under test. The reaction was terminated by addition of 100 μ l Stop mix (50 μ M ATP, 5 mM EDTA, 0.1% BSA, 0.1% Triton X-100 and 7.5 mg/ml Streptavidin coated PVT SPA beads). After allowing the beads to settle for 30 minutes, the assay mixture

was counted in a microtiterplate scintillation counter. The results are illustrated in Figure 7.

5 AKT3 filter assays were performed at 25°C for 3 hrs in the presence of 25mM Hepes, pH7.0, containing 15 mM MgCl₂, 1 mM DTT. Each assay was performed in a 100 µl reaction volume containing 111 nM GST-AKT-3 (diluted in 25mM Hepes, pH7.0, containing 15 mM MgCl₂, 1 mM DTT), 2.5 µM Histone H2B, 2nM [γ-³³P]-ATP and any
10 agents under test. The reaction was terminated by addition of 100 µl 75 mM H₃PO₄. 90µl of the assay mixture was filtered through Phosphocellulose cation exchange paper. After five times washing with 75 µM H₃PO₄, the filterpaper was counted in a
15 microtiterplate scintillation counter. The results are illustrated in Figure 8.

RESULTS

20 Molecular cloning of human Akt-3.

Similarity searching of the LifeSeq™ and EMBL databases using the rat Akt-3 sequence as a query sequence yielded several human EST sequences which
25 encoded part of the human homologue of rat Akt-3. Using the DNA sequence information in the databases, we were able in subsequent 3' RACE experiments to deduce the complete cDNA sequence for the human Akt-3 (Figure 1). The obtained cDNA sequence encoded a
30 protein of 479 amino acid residues with a calculated molecular mass of 55770 Da. The first 451 amino acids of the human Akt-3 protein contain only two differences to the corresponding rat sequence (Konishi et al., 1995) - Asp (rat) to Gly (human) at position
35 10 and Pro (rat) to Ala (human) at position 396 and encode a pleckstrin homology domain, a kinase domain

- 27 -

and a COOH-terminal "tail". The predicted Akt-3 (Figure 2) protein shows significant similarity with Akt-1 (Jones et al, 1991; 83.6% identity; 87.8% similarity) and with Akt-2 (Cheng et al., 1992; 78% identity; 84.3% similarity). The COOH-terminal 'tail' has been observed in both human and rat Akt-1 and Akt-2 proteins, but it is apparently truncated in the only other reported Akt-3 sequence (rat Akt-3, Konishi et al., 1995; accession number D49836). 3'RACE experiments performed on human cDNAs derived from different tissues did not yield evidence for the existence of a shorter form of Akt-3 that would be analogous to the rat Akt-3 (data not shown). The tail in human Akt-3 comprises 28 amino acid residues (YDEDGMDCMDNERRPHFPQFSYSASGRE) that replace 3 amino acid residues in the rat sequence (CPL). The tail in human Akt-3 contains a serine residue at position 472 (shown in bold) that corresponds to Ser⁴⁷³ in Akt-1 or Ser⁴⁷⁴ in Akt-2. Phosphorylation of Ser⁴⁷³ and Ser⁴⁷⁴ has previously been implicated in the activation of Akt-1 and Akt-2, respectively (Alessi et al., 1996; Meier et al., 1997). Thr³⁰³ (in the kinase domain) has also been implicated in the activation of Akt-1 and this residue is also conserved in human Akt-3 (Thr³⁰⁵).

Characterisation of Akt-3 activity.

To characterise the enzymatic activity of Akt-3, we expressed and purified the recombinant enzyme as a GST fusion protein. Analysis of the purified product by SDS-PAGE indicated the protein was apparently > 90% pure. The purified enzyme was able to phosphorylate histone H2B (figure 3), and no phosphorylation was observed using recombinant GST alone. Previously, the enzymatic activity of Akt-1 has been shown to be increased by phosphorylation of Thr³⁰³ and Ser⁴⁷³, and

mutation of both these residues to Asp (to mimic phosphorylation) synergistically activates Akt-1 (Alessi et al., 1996). To investigate whether Akt-3 is similarly regulated, GST-fusion proteins in which
5 either Thr³⁰⁵ or Ser⁴⁷² (corresponding to Thr³⁰⁸ and Ser⁴⁷³ in Akt-1) or both Thr³⁰⁵ and Ser⁴⁷² had been mutated to Asp were expressed and assayed in comparison to the wild-type enzyme. Mutation of Thr³⁰⁵ to Asp ("T305D") resulted in a 2.0-fold increase in the initial rate of
10 phosphorylation of histone H2B, whereas mutation of Ser⁴⁷² to Asp("S472D") increased the initial rate only 1.4 fold (Figure 3A). When both Thr³⁰⁵ and Ser⁴⁷² ("T305D,S472D") were mutated to Asp, a 3.2-fold increase in the initial phosphorylation rate was
15 observed.

To confirm that extracellular stimuli can activate Akt-3 in mammalian cells, Cos-7 cells were transfected with a cDNA encoding Akt-3 fused to a HA tag. Akt-3
20 activity in HA immunoprecipitates was increased 1.5 and 1.9 fold (n=2) following stimulation with IGF-1 (10 ng/ml).

To further confirm that extracellular stimuli can
25 activate Akt-3 in mammalian cells, HEK-293 cells were transfected with a cDNA encoding Akt-3 fused to a HA epitope tag. Upon treatment with IGF, Akt-3 activity in anti-HA immunoprecipitates (Figure 3B) was increased almost 60-fold above that in untransfected
30 cells (Figure 3C). Akt variants in which Thr³⁰⁵ and Ser⁴⁷² were mutated to alanine were refractory to activation by IGF. Consistent with this, Western blotting with a Ser⁴⁷² phosphospecific antibody of HA immunoprecipitates from cells stimulated with IGF
35 demonstrated that Ser⁴⁷² was phosphorylated following stimulation with IGF (Figure 3B). In addition, activation of Akt-3 was inhibited by prior treatment

with CY29 4002 (100 μ M, 94% inhibition), data not shown).

5 To characterise human Akt-3 further, we investigated the ability of a range of Ser/Thr kinase inhibitors to inhibit Akt-3. These included Go 6976, GF-109203X (both protein kinase C (PKC) inhibitors); H-85, H-88, H-89 and KT5720 (protein kinase A (PKA) inhibitors), KN-62 (Ca^{+2} /Calmodulin dependent kinase inhibitor) and
10 PD 98059 (MEK inhibitor). When tested at a concentration of 10 μ M these compounds had no significant effect on the activity of the T305D,S472D variant of Akt-3. However, the broad spectrum kinase inhibitor staurosporine (IC_{50} = $2.0 \pm 0.3 \mu\text{M}$) and the
15 PKC inhibitor Ro 31-8220 (IC_{50} = $3.2 \pm 1.0 \mu\text{M}$) inhibited the T305D,S472D variant of Akt-3 (Figure 4).

Chromosomal localisation of Akt-3.

20 The complete coding sequence of Akt-3 was used as a probe for FISH analysis. Under the conditions used, the hybridisation efficiency was approximately 75% for this probe (among 100 checked mitotic figures, 75 of them showed signals on one pair of the chromosomes).
25 Since the DAPI-banding was used to identify the specific chromosome, the assignment between the signal from the probe and the long arm of chromosome 1 was obtained. The detailed position was further determined in the diagram based upon the summary from 10
30 photographs (Figure 5A). There was no additional locus picked by FISH detection under the conditions used, therefore, it was concluded that Akt-3 is located at human chromosome 1, region q43-q44. An example of the mapping results is presented in Figure 5B.

35

Tissue distribution of Akt-3 mRNA.

Northern blot analysis was performed on mRNA derived from different human tissues. Akt-3 mRNA was detected as two transcripts of approximately 4.5 kb and 7.5 kb, showing similar patterns of expression (Fig. 6A). Akt-3 mRNA was expressed in a range of tissues, most prominently in brain. Similarly, rat Akt-3 was detected as multiple transcripts most highly expressed in brain (Konishi et al., 1995). The weakest expression of Akt-3 was observed in two insulin-responsive tissues, skeletal muscle and liver. Akt-3 was also expressed in a number of cancer cell lines including SW480 colorectal adenocarcinoma, A549 lung carcinoma and G361 Melanoma (data not shown).

To confirm the Northern blot analysis, PCR reactions were performed with Akt-3 specific and G3PDH-specific (internal control) primers on cDNAs derived from different human tissues (Fig. 6B). The Akt-3 message was present in every tissue tested, since a specific 425 bp fragment was amplified in every cDNA after 30 cycles of PCR. Akt-3 mRNA expression was high in placenta, ovary and spleen. Moderate expression was seen in brain, heart, kidney, colon, prostate, small intestine and testis. Lowest expression was in liver, lung, pancreas, skeletal muscle, peripheral blood leukocytes and thymus. In tumor cell lines (Figure 6C), Akt-3 mRNA expression was relatively high in HT-1080 bone fibrosarcoma cells, in SaOS-2 osteosarcoma and in JURKAT T-cell leukemia cells (Akt-3 band detectable after 30 cycles of PCR). Caco-2 colorectal adenocarcinoma, T84 colorectal carcinoma, MCF-7 breast adenocarcinoma and SK-N-MC neuroblastoma cells show Akt-3 mRNA expression after 35 cycles of PCR. In T-47D breast ductal gland carcinoma and HepG2 hepatoblastoma, expression of Akt-3 mRNA is very low or absent (no signal detectable after 35 cycles of PCR).

Akt-1 and Akt-2 have been identified in several species. Human (Jones et al., 1991; Coffe et al 1991), mouse (Bellacosa et al., 1993) and bovine (Coffe & Woodgett, 1991) Akt-1 clones have been reported, whereas human (Cheng et al., 1992) mouse (Altomare et al., 1995) and rat (Konishi et al., 1994) clones of Akt-2 have been identified. However, Akt-3 has only been previously identified in rat (Konishi et al, 1995). The present inventors have identified the human isoform of Akt-3. Although human Akt-3 shows considerable similarity to human Akt-1 and Akt-2, the discovery of human Akt-3 is particularly significant because the cDNA sequence encodes a COOH-terminal "tail" which includes a phosphorylation site implicated in the activation of Akt-1 and Akt-2 (Alessi et al., 1996; Meier et al., 1997). This tail is absent from the predicted rat amino acid sequence. Human Akt-3 appears to be activated by phosphorylation in a similar fashion as Akt-1 and Akt-2. However, its expression profile suggests that the principal function of this enzyme is not in regulating responses to insulin.

The sequence which has been identified represents the human homologue of Akt-3. This assignment is based on the >99% identity between the rat and human Akt-3 protein sequences. With the exception of the COOH-terminal tail seen in human Akt-3, there are only 2 amino acid differences (Gly¹² and Ala³⁹⁶ in human Akt-3) between the rat and human Akt-3 proteins. Alignment of all the previously described Akt sequences demonstrates that Gly¹⁰ and Ala³⁹⁶ in the human protein correspond to Gly and Ala residues respectively in the Akt-1 and Akt-2 sequences identified from other species. Further evidence that we have identified the Akt-3 isoform comes from the presence of isotype-specific sequences represented by human Akt-3 residues

47-49 (LPY), 118-122 (NCSPT) and 139-141 (HHK). For each isotype, these sequences are conserved between species, but differ between the isotypes.

5 The human Akt-3 cDNA sequence was predicted to encode a NH₂-terminal pleckstrin homology (PH) domain (Musacchio et al., 1993) and a COOH-terminal kinase domain. A striking difference between the human and
10 rat Akt-3 protein sequence (Konishi, et al., 1995) is the presence of a COOH-terminal "tail" comprising 74 residues after the kinase domain. The last 28 amino acid residues in human Akt-3 are absent from the rat Akt-3 sequence. We were unable to identify human cDNA
15 sequences that encoded a similar truncation, despite conducting RACE experiments using cDNA from several different human tissues. The region in human Akt-3 that is absent from rat Akt-3 encompasses a stretch of 10 residues (residues 467-476 in human Akt-3) which are identical to the corresponding region of human
20 Akt-1 and Akt-2. This suggests that the tail observed in human Akt-3 is authentic. The significance of the difference observed in the rat Akt-3 tail region remains to be investigated. However, the human Akt-3 COOH-terminal sequence includes Ser⁴⁷², which
25 corresponds to Ser⁴⁷³ in Akt-1. Phosphorylation of Ser⁴⁷³ has been shown to lead to a 5-fold increase in the activity of Akt-1, whereas a 20-25 fold increase of Akt-1 activity is observed if both Ser⁴⁷³ and Thr³⁰³ are phosphorylated (Alessi et al., 1996). Thus, our
30 observation that Ser⁴⁷² is present in human Akt-3 is significant, because it suggests that human Akt-3 is potentially regulated in a manner similar to Akt-1 and Akt-2. Whether rat Akt-3 is regulated in a different fashion remains to be resolved.

35

The kinase and PH domains in Akt-3 show homology to the consensus PH and kinase domain sequences

(Musacchio et al., 1993; Hanks & Hunter 1995). The PH domain of human Akt-3 is 77% and 86% identical to the PH domains in Akt-1 and Akt-2, respectively, while the kinase domain of Akt-3 is 88% and 87% identical to the kinase domain of Akt-1 and Akt-2, respectively. The high conservation of the PH domain may indicate an Akt-specific function, because PH domains are often highly divergent (Musacchio et al., 1993). Apart from binding phosphoinositides, the PH domain of Akt has been shown to mediate interactions between Akt and PKC (Konishi, et al., 1995) as well as directing the formation of multimeric Akt complexes (Datta et al., 1995). In contrast, the region between the PH domain and the kinase domain is poorly conserved between the human Akt-1, Akt-2 and Akt-3 sequences, and this region is also important for mediating the formation of multimeric Akt complexes (Datta et al., 1995). This raises an interesting issue - whether the sequence NH₂-terminal to the kinase domain of Akt-3 mediates the interaction with binding partners that are unique to Akt-3 or that bind to multiple Akt isoforms.

To verify that the predicted kinase domain was catalytically active, we expressed Akt-3 as a GST fusion protein in *E. coli*. The purified protein was able to phosphorylate an exogenous substrate, whereas no catalytic activity was observed using GST in place of GST-Akt-3. To confirm that Akt-3 is indeed regulated in a manner akin to Akt-1 and Akt-2, we mutated Thr³⁰³ and Ser⁴⁷³, either separately or jointly, to Asp. This strategy has previously been shown to faithfully mimic the effect of phosphorylation of these residues in Akt-1 (Alessi et al., 1996). Mutation of either of these residues resulted in increased activity, although the increase was less than that observed with Akt-1 (Alessi et al., 1996). Additionally, we did not observe a synergistic

activation of Akt-3 by mutation of both Thr³⁰⁵ and Ser⁴⁷³. In contrast, when both the corresponding residues were simultaneously mutated to Asp in Akt-1, synergistic activation was observed (Alessi et al, 1996). The apparent quantitative differences between Akt-1 and Akt-3 may reflect true differences in the regulation of these two isoforms, or it may be due to other factors such as the different expression system used. In the present study Akt-3 was expressed as a GST fusion protein in *E. coli*, whereas Akt-1 activity was studied using an HA-tagged protein expressed in COS cells. Nevertheless, our results demonstrate that Akt-3 is qualitatively regulated in a fashion similar to Akt-1. Previous work has also shown that activation of Akt is dependent upon PI 3-kinase to generate 3-phosphoinositides that bind the PH domain of Akt, promote translocation of Akt to the plasma membrane and facilitate the phosphorylation of Akt by upstream kinases (reviewed in Alessi & Cohen, 1998; Coffey et al., 1998). Our observation that the T305D/S472D mutant of Akt-3 is more active than the wild type enzyme (Figure 3), when measured in the absence of 3-phosphoinositides, suggests that after phosphorylation Akt-3 becomes (at least partially) independent of phosphoinositide binding.

The structure of the catalytic domain of Akt is closely related to protein kinase A and protein kinase C. Indeed, a BLAST search of the SwissProt data base revealed that the most closely related kinases (other than the different Akt isoforms) include several protein kinase C isozymes. This prompted us to investigate whether existing inhibitors of PKA or PKC, as well as other serine/threonine kinase inhibitors, could be used as inhibitors of Akt-3. Of the compounds tested, only staurosporine and the structurally related compound Ro 31-8220 both potently inhibited

Akt-3. Staurosporine is a non-selective kinase inhibitor, whereas Ro 31-8220 is a more selective PKC inhibitor (Davis, et al., 1992). Although Ro 31-8220 is an approximately 100-fold more potent ($IC_{50} \approx 10$ nM; Davis, et al., 1992) inhibitor of PKC than of Akt-3, this observation cautions that experiments using high concentrations of Ro 31-8220 may affect Akt-3. In contrast to staurosporine and Ro 31-8220, two other PKC inhibitors and three other PKA inhibitors did not inhibit Akt-3. This suggests that although Akt-3 is closely related in sequence to PKC, it may be possible to find selective inhibitors of Akt.

The observation that Akt-3 is activated by IGF-1 suggests that Akt-3 may play a role in regulating cell survival. Akt-3 potentially may suppress apoptosis in tumor cells. One concern in using Akt as a target for drug development in cancer is that Akt plays a role in insulin signalling (reviewed in Sheperd et al, 1998). Thus, inhibitors of Akt may induce symptoms observed in patients with diabetes. One solution that has been proposed is to develop selective inhibitors of Akt-2 (Walker et al, 1998). This is based in part on the observation that Akt-1 is strongly activated by insulin in rat hepatocytes and skeletal muscle, whereas Akt-2 is only weakly activated by insulin in these tissues. However, rat Akt-3 appears to be even more weakly activated by insulin in these tissues (Walker et al, 1998), and in this study we have shown that Akt-3 mRNA is expressed only at low levels in human liver and skeletal muscle, which are insulin responsive tissues. This suggests that selective inhibitors of Akt-3 could have even less potential to cause symptoms similar to those seen in patients with diabetes than do inhibitors of Akt-2. The localisation of human Akt-3 to human chromosome 1q43-44 is also interesting, as patients with haematological cancers

have been reported with chromosomal abnormalities in this region (Mitelman et al, 1997). Although the significance of the latter observation is debatable, as chromosomal abnormalities at numerous loci have been observed in patients with haematological cancers, the results presented here indicate that Akt-3 may prove to be an important target for the development of novel therapeutics for the treatment of cancer.

SEQUENCE LISTING

1. Sequence ID No. 1 corresponds to the nucleotide
sequence of Akt-3 illustrated in Figure 1.
- 5 2. Sequence ID No. 2 corresponds to from nucleotide
position 11 to 1447 of the nucleic acid sequence
of Akt-3 illustrated in Figure 1.
- 10 3. Sequence ID No. 3 corresponds to the amino acid
sequence of Akt-3 illustrated in Figures 1 and 2.

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- 25

CLAIMS

1. A nucleic acid molecule encoding human Akt-3 protein or a functional equivalent, derivative or bioprecursor thereof, comprising the amino acid sequence illustrated in SEQ ID No. 3.
2. A nucleic acid molecule according to claim 1 which is a DNA molecule, and preferably cDNA.
3. A nucleic acid molecule according to claim 1 or 2 comprising the nucleotide sequence illustrated in SEQ ID No. 1.
4. A nucleic acid molecule according to claim 1 or 2 comprising the nucleotide sequence in SEQ ID No. 2.
5. An antisense molecule capable of hybridising to the molecule according to any of claims 1 to 4 under high stringency conditions.
6. A human Akt-3 protein or a functional equivalent, derivative or bioprecursor thereof, comprising an amino acid sequence as illustrated in SEQ ID No. 3.
7. A human Akt-3 protein or a functional equivalent, derivative or bioprecursor thereof encoded by a nucleic acid molecule according to any of claims 1 to 4.
8. A human Akt-3 protein according to claim 7 comprising the amino acid sequence as illustrated in SEQ ID No. 3.
9. An expression vector comprising a nucleic acid molecule according to claim 2 or 3.

10. An expression vector according to claim 9 comprising an inducible promoter.
- 5 11. An expression vector according to claim 9 or 10 comprising a sequence encoding a reporter molecule.
12. A nucleic acid molecule according to any of claims 1 to 5 for use as a medicament.
- 10 13. Use of a nucleic acid molecule according to any of claims 1 to 5 in the preparation of a medicament for treating cancer.
- 15 14. A human Akt-3 protein according to any of claims 6 to 8 for use as a medicament.
15. Use of a human Akt-3 protein according to any of claims 6 to 8 in the preparation of a medicament for treating cancer.
- 20 16. A pharmaceutical composition comprising a nucleic acid molecule according to any of claims 1 to 5 or a human Akt-3 protein according to any of claims 6 to 8 together with a pharmaceutically acceptable carrier
- 25 diluent or excipient therefor.
17. A host cell or organism, transformed or transfected with an expression vector according to any of claims 9 to 11.
- 30 18. A transgenic cell, tissue or organism comprising a transgene capable of expressing a human Akt-3 protein according to any of claims 6 to 8.
- 35 19. A human Akt-3 protein expressed from the cell or organism according to claim 17 or 18.

20. An antibody capable of binding to a human Akt-3 protein or an epitope thereof according to any of claims 6 to 8.

5 21. An antibody according to claim 20 which is a monoclonal antibody.

22. An antibody according to claim 20 or 21 for use as a medicament.

10

23. Use of an antibody according to claim 20 or 21 in the preparation of a medicament for treating cancer, or other diseases or conditions associated with human Akt-3 protein expression.

15

24. A kit for detecting human Akt-3 protein in a sample which protein comprises a sequence according to any of claims 6 to 8, said kit comprising an antibody according to claim 20 or 21 and means for contacting
20 said antibody with said sample.

25. A method of identifying compounds which selectively inhibit human Akt-3 protein mediated promotion of cell survival said method comprising:

25

i) providing a cell transformed with an expression vector activating the Akt-3 pathway that survives in the presence or absence of a survival factor compared to a control cell which has not been transformed with said vector and will die in the absence of said survival factor,

30

ii) contacting each of said cells with a test compound following the removal of said cells from said survival factor, wherein death of said transformed cell is indicative of selective inhibition of said compound on the survival promoting human Akt-3 pathway.

35

26. A method of identifying compounds which selectively inhibit human Akt-3 protein mediated promotion of cell survival, said method comprising:

- 5 i) providing a cell transformed with an expression vector activating the Akt-3 pathway in addition to a control cell which has not been transformed with said vector,
- 10 ii) contacting each of said cells with a death inducing agent, whereby death of said control cell and survival of said transformed cell is indicative of the survival promoting activity of the activated Akt-3 pathway,
- 15 iii) contacting said transformed cell with a test compound, wherein death of said cell is indicative of selective inhibition of said compound on the survival promoting human Akt-3 pathway.

20 27. A compound identifiable according to the method of claims 25 or 26.

25 28. A compound according to claim 27 for use as a medicament.

29. Use of a compound according to claim 27 in the manufacture of a medicament for treating diseases associated with human Akt-3 protein expression.

30 30. A method of identifying agents which influence the activity of a human Akt-3 protein according to any of claims 6 to 8, said method comprising contacting said human Akt-3 protein with a substrate therefor in the presence of a test compound and a phosphate
35 source, and monitoring for any phosphorylation of said substrate.

31. A method according to claim 30 wherein said Akt-3 protein is provided as a fusion or epitope tagged protein having a domain capable of phosphorylating a known substrate.

5

32. A method according to claim 30 wherein said Akt-3 protein is provided as a fusion molecule of GST and human Akt-3.

10

33. A method of identifying agents which influence the activity of a human Akt-3 protein according to any of claims 6 to 8, said method comprising contacting a phospholipid or a surrogate or functional equivalent thereof, with a PH domain of a human Akt-3 protein according to any of claims 6 to 8 in the presence of an agent to be tested and monitoring for any binding of said phospholipid, surrogate or functional equivalent thereof with said PH domain of said Akt-3 protein.

20

34. A method according to claim 33 wherein said phospholipid comprises phosphatidylinositol 3,4,5-triphosphate.

25

35. An agent identifiable according to the method of claim 33 or 34.

36. An agent according to claim 35 for use as a medicament.

30

37. Use of an agent according to claim 35 in the preparation of a medicament for treating diseases associated with human Akt-3 expression.

35

38. A method of treating diseases associated with human Akt-3 activity said method comprising administering to an individual suffering from said

disease a compound that inhibits the function and/or expression of a human Akt-3 protein according to any of claims 6 to 8, in a sufficient concentration to reduce the symptoms of said disease.

5

39. A method according to claim 38 wherein said compound is any of an antisense molecule according to claim 5, an antibody according to claim 21 or 22, a compound according to claim 27 or an agent according to claim 35.

10

40. A method for making a pharmaceutical formulation for the treatment of diseases associated with human Akt-3 protein expression, said method comprising:

15

- a) contacting candidate compounds with a host cell that expresses human Akt-3 protein,
- b) selecting a compound identified in step a) which binds to human Akt-3 protein,
- c) manufacturing bulk quantities of the compound selected in step b), and
- d) formulating the compound manufactured in step c) in a pharmaceutically acceptable carrier.

20

25

41. A method of identifying a compound which modulates Akt-3 kinase activity, comprising:

- a) contacting said Akt-3 with a substrate thereof in the presence of a radiolabelled phosphate source, and the compound to be tested,
- b) stopping the reaction by the addition of kinase inhibitor in the presence of SPA beads,
- c) monitoring the signal from said beads compared to a control which has not been contacted with said compound.

30

35

42. A method of identifying a compound which

modulates Akt-3 activity, comprising:

- 5 a) contacting said Akt-3 with a substrate
 thereof in the presence of a radiolabelled
 phosphate source, and the compound to be
 tested,
- b) stopping the reaction,
- c) filtering the reaction mixture through
 phosphocellulose cation exchange paper, and
- 10 d) monitoring the signal from said filter paper
 compared to a control which has not been
 contacted with said compound.

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FIG. 1.

1 GGGAGTCATCATM S D V T I V K E G W V O K R G E 17
61 ATATATAAAAACTGGAGGCCAAGATACTTCTTTTGAAGACAGATGGCTCATTTCATAGG 37
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361 GAATTGTAGTCCAACCTTCACAAATTGATAATATAGGAGAGGAAGAGATGGATGCCTCTAC 137
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1321 AGAATTTACAGCTCAGACTATTACAAATAACACCACCTGAAAAATATGATGAGGATGGTAT 457
1381 D C M D N E E E E H E P Q F S Y S A S G 477
1441 P E ACGAGATAAGTCTCTTTCATTCCTGCTACTTCACTGTCTCTTCAATTTTACTGAAAA 479
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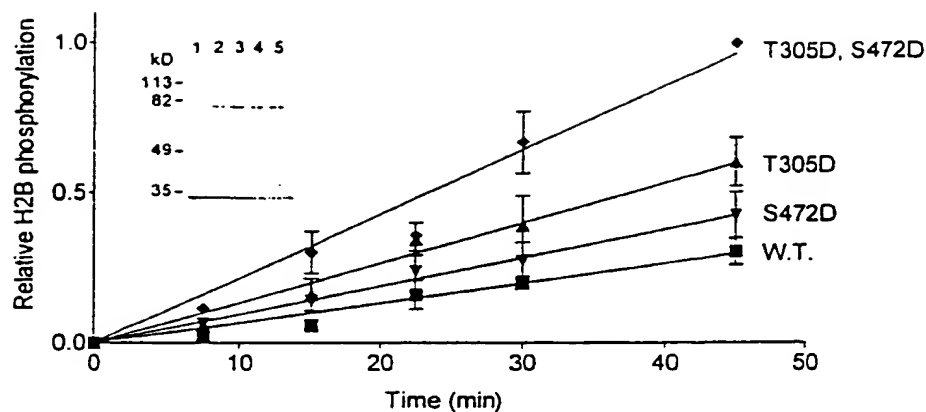
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akt-2	UTPP	GRD	---	LGLE	DOR	MEPQSY	SAS	TA	: 481			
akt-3	UTPP	EK	DE	EDG	D	M	H	SR	MEPQSY	SAS	TA	: 479

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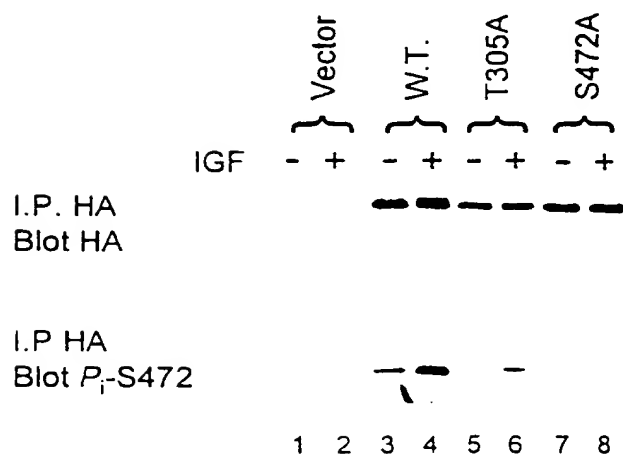
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FIG. 3.

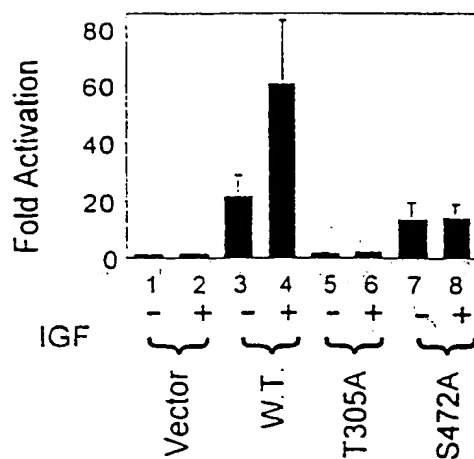
A



B



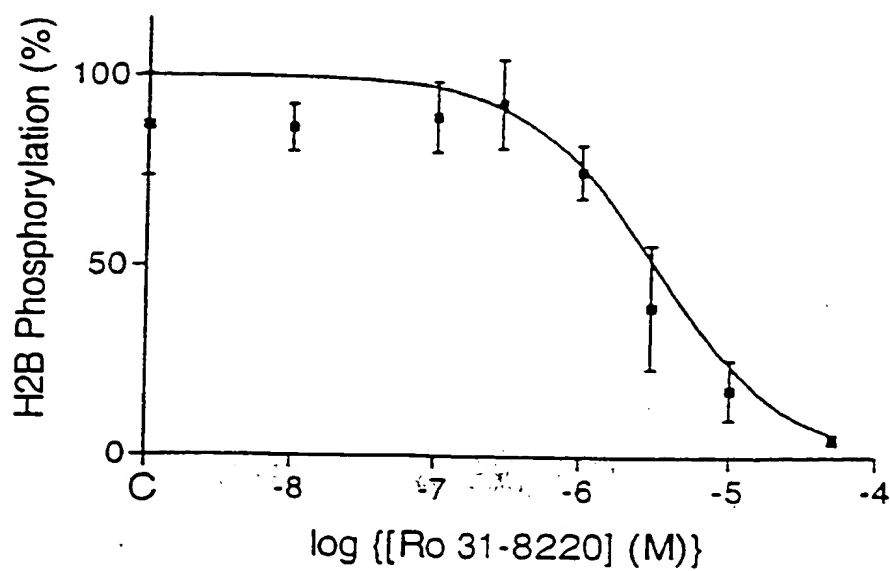
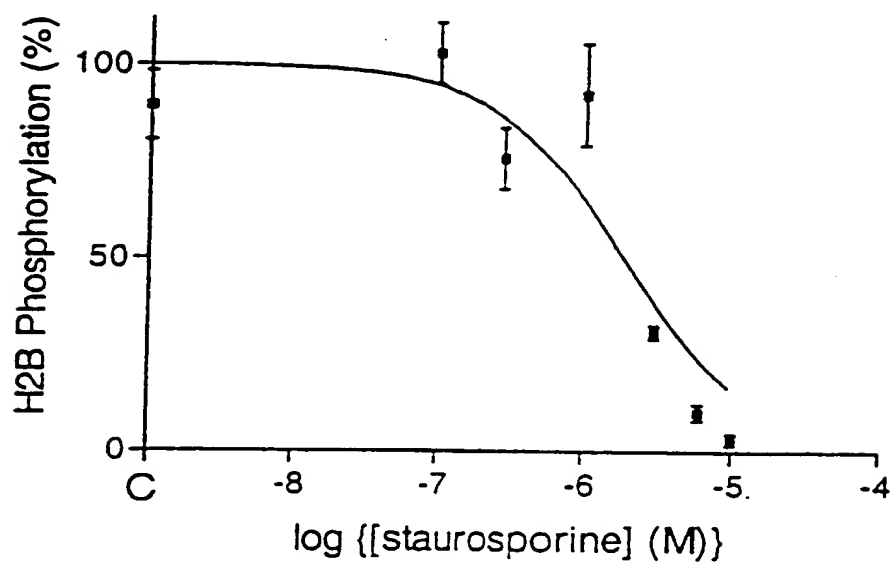
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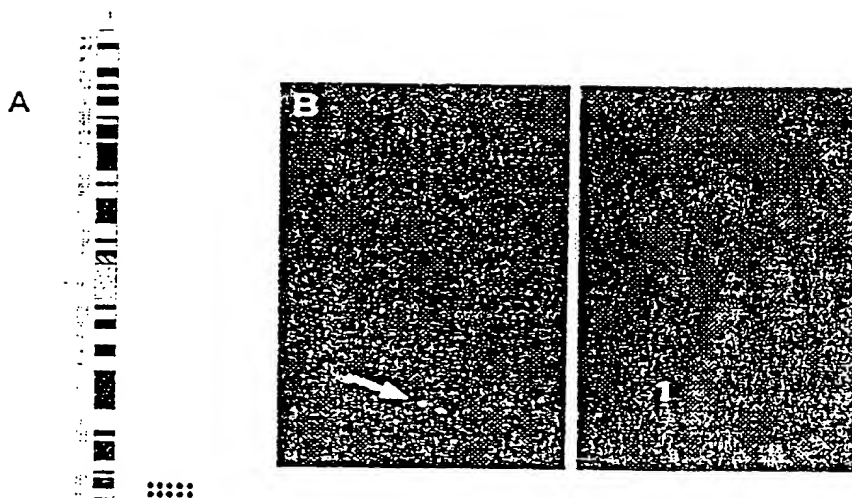
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FIG. 4.



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FIG. 5.



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FIG. 6A.

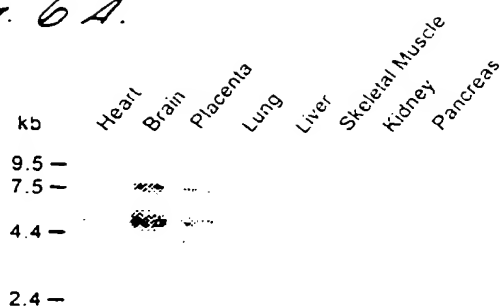


FIG. 6B.

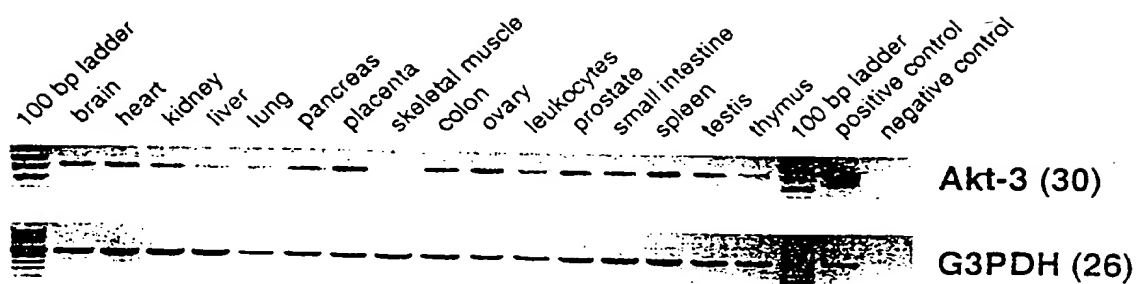
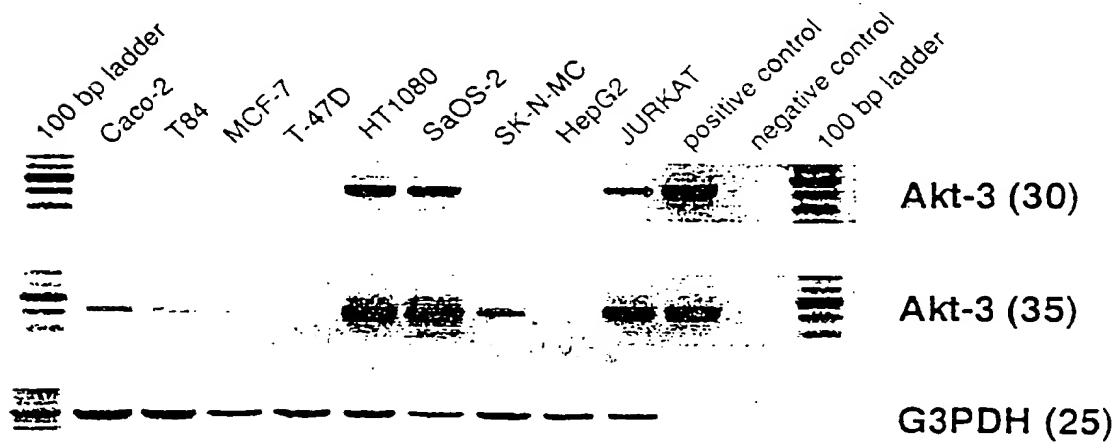
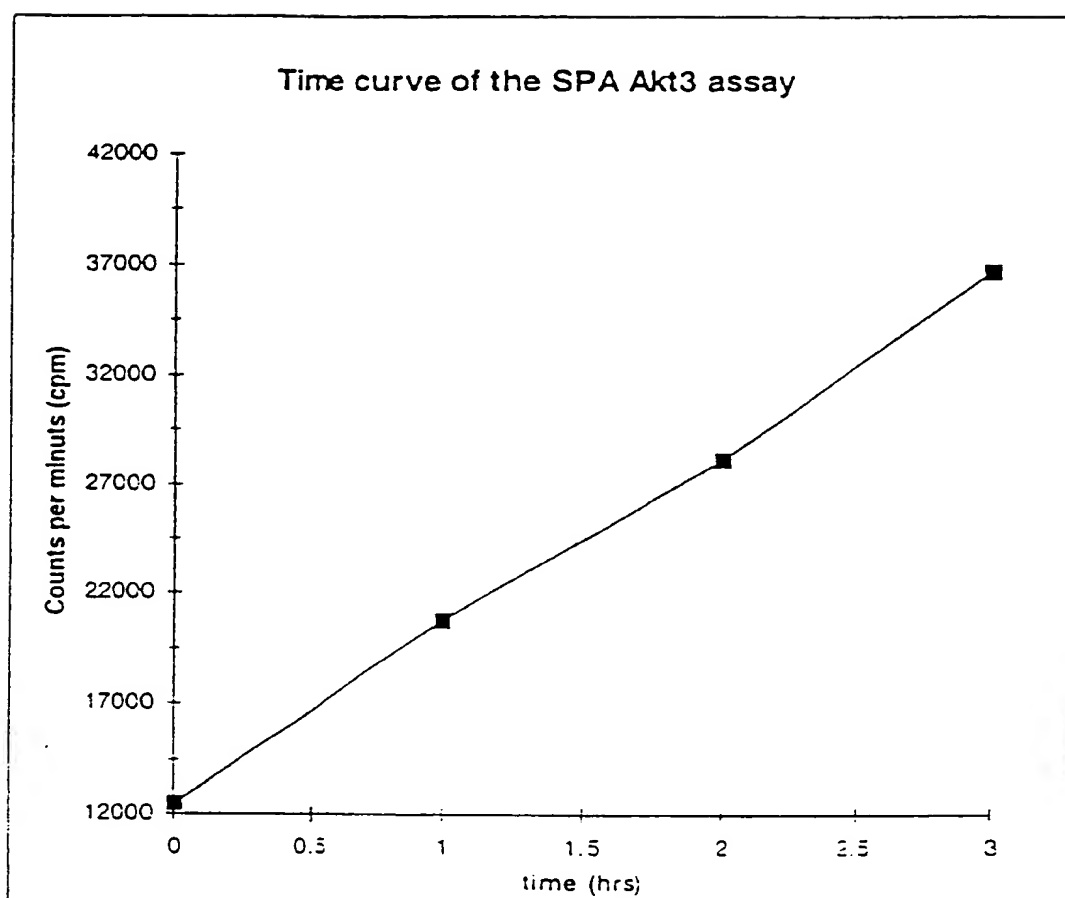


FIG. 6C.

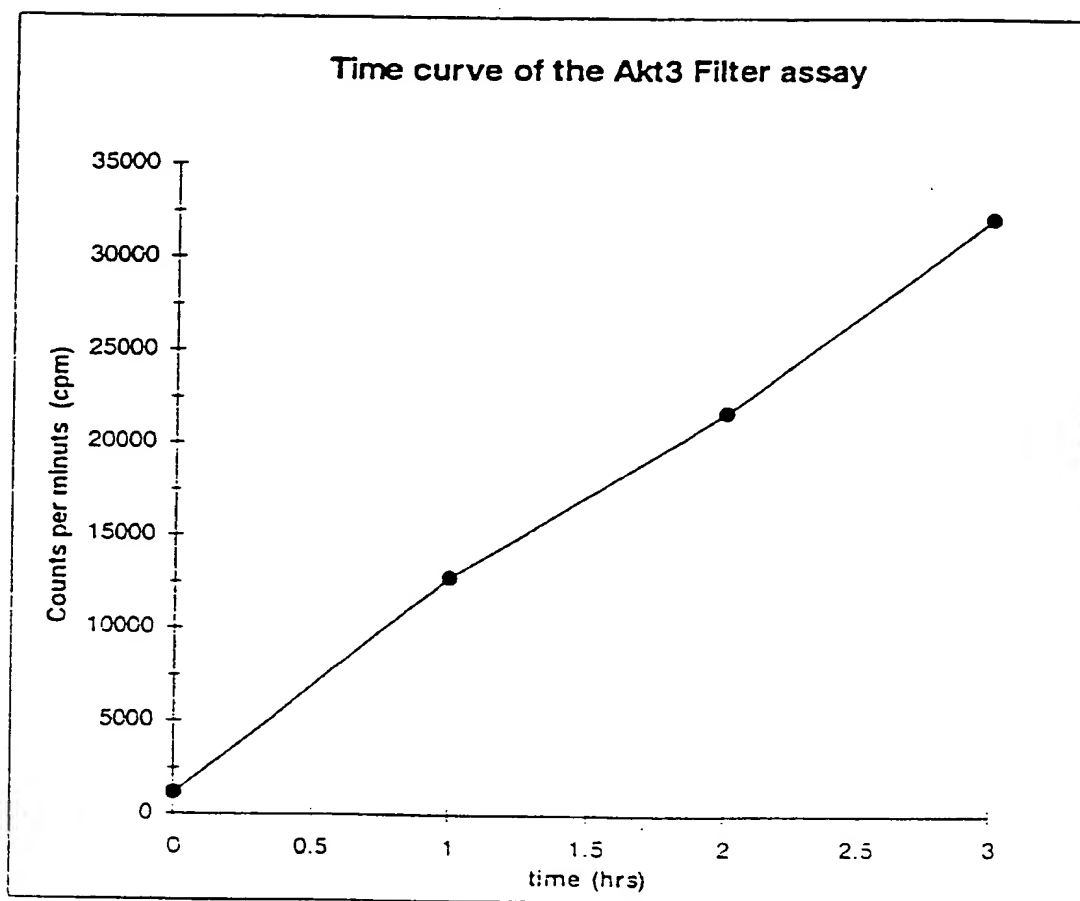


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FIG. 7

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FIG. 8.

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 99/04311

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C12N15/54	C12N9/12	C12N15/10	C12N15/70	C12N15/85
	C12N5/10	C07K16/40	C12Q1/48	A61K38/45	A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, STRAND, MEDLINE, BIOSIS, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MASURE, S. ET AL.: "Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 265, 1999, pages 353-360, XP000917167 the whole document	1-11, 16-19
P,X	BRODBECK, D. ET AL.: "A human protein kinase B gamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 14, 2 April 1999 (1999-04-02), pages 9133-9136, XP000919262 the whole document	1-4, 6-11, 16-19
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

1 August 2000

Date of mailing of the international search report

28.08.00

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/04311

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WALKER, K.S. ET AL.: "Activation of protein kinase B β and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha" THE BIOCHEMICAL JOURNAL, vol. 331, no. 1, 1 April 1998 (1998-04-01), pages 299-308, XP000917147 cited in the application the whole document	20
Y		1-11, 16-19, 21, 23, 24
Y	KONISHI, H. ET AL.: "Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and B/gamma subunits of G proteins " BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 216, no. 2, 13 November 1995 (1995-11-13), pages 526-534, XP002030406 cited in the application the whole document	1-26, 30-34, 41
Y	ALTOMARE, D.A. ET AL.: "Cloning, chromosomal localization and expression analysis of the mouse Akt2 oncogene" ONCOGENE, vol. 11, no. 6, 21 September 1995 (1995-09-21), pages 1055-1060, XP000917027 cited in the application the whole document	1-11, 16-21, 23, 24
Y	WO 97 18303 A (NOVARTIS AG) 22 May 1997 (1997-05-22) page 4, paragraph 3 -page 13, paragraph 2	1-26, 30-34, 41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/04311

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 27-29, 35-40
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 27-29,35-40

Claims 27-29 and 35-37 concern a compound or agent and the use thereof in the preparation of a medicament. The compound or agent is only defined by the method which can be used in order to identify said compound or agent, respectively. Claims 38-40 concern a method comprising a compound or agent according to claims 27-29 and 35-37, or which method comprises a compound which is only defined in that it inhibits the function and/or expression of a human Akt-3 protein, and in that it binds to human Akt-3 protein, respectively. Since it is completely unclear which kind of substances will be identified by the respective methods and since in the specification no concrete examples for these kind of substances are given, the scope of said claims is totally ambiguous and undefined. Moreover, it cannot be excluded that even substances known in the art may be recognized as agents that influence or inhibit the function and/or expression of human Akt-3 protein.

In view of the wording of the claims presently on file, which render it difficult, if not impossible, to determine the matter for which protection is sought, the present International application fails to comply with the clarity requirements of Article 6 PCT to such an extent that a meaningful search of the claims is impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear, namely claims 1-26, 30-34 and 41.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

Information on patent family members

PCT/99/04311

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9718303 A	22-05-1997	AU 7564396 A	05-06-1997
		EP 0862622 A	09-09-1998
		JP 2000500746 T	25-01-2000

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